INFECTION AND IMMUNITY, June 1989, p. 1680–1683
0019-9567/89/061680-04$02.00/0

Vol. 57, No. 6

Fixation of *Clostridium difficile* Toxin A and Cholera Toxin to Intestinal Brush Border Membranes from Axenic and Conventional Mice

F. LUCAS,[1] G. W. ELMER,[2] E. BROT-LAROCHE,[3] AND G. CORTHIER[1]*

Laboratoire d'Ecologie Microbienne, Institut National de la Recherche Agronomique, CRJ 78350 Jouy-en-Josas, France[1]; Department of Medical Chemistry, University of Washington, Seattle, Washington 98195[2]; and Centre de Recherches sur la Nutrition, Centre National de la Recherche Scientifique, Meudon, France[3]

Received 21 November 1988/Accepted 3 March 1989

We have tested the in vitro binding of *Clostridium difficile* toxin A (enterotoxin) and cholera toxin to intestinal brush border membranes prepared from either conventional or axenic mice. Membranes from axenic mice were shown to be saturated at a lower toxin A concentration (at least 2.5 times lower). Because there were no significant differences between membranes from axenic and conventional mice in binding at low toxin A concentrations, the presence of the normal microflora seems to increase the number but not the affinity of brush border membrane receptors on the enterocyte surface. Corroborating the in vitro results, we observed that conventional mice were more sensitive to the pathological effects of toxin A given intragastrically than were axenic mice. In contrast, there was no difference in the binding characteristics of cholera toxin between membranes from conventional and axenic mice. We conclude that the presence of the mouse intestinal bacteria increases the number of *C. difficile* toxin A intestinal receptors but does not influence cholera toxin receptors.

It is now well established that *Clostridium difficile* is responsible for causing pseudomembranous colitis, a severe manifestation characterized by the presence of colonic membranes, hemorrhagic lesions, and profuse watery diarrhea (1, 10, 11). In healthy individuals, the microbial flora of the digestive tract exerts a preventive action against the establishment and proliferation of *C. difficile*. Patients treated with antibiotics, however, are especially at risk for this infection. The infection can be experimentally produced by inoculating clindamycin-treated hamsters (2, 4, 8, 19) or axenic mice (5, 17) with *C. difficile*, and these two models have contributed much to the comprehension of *C. difficile*-associated pathology. Toxin A (enterotoxin) is recognized to be highly important in the pathological process by inducing water loss (15, 18, 20). The role of toxin B (cytotoxin) is less clear but may act synergistically with toxin A to create the characteristic damage to the intestinal and colonic epithelia (16). More recently, Krivan and co-workers have described specific intestinal receptors for *C. difficile* toxin A from hamsters and rats (13) which are apparently different from the GM1 receptor described for cholera toxin (9). Binding was found to be greater to hamster receptor preparations than to those of rats, an animal of low sensitivity to toxin A. These findings strongly suggest that *C. difficile* disease is mediated by binding of toxin A to specific receptors and that the affinity and number of these receptors may be important in the expression of pathology. Factors that modify the structure and function of the receptors are therefore of interest. The purpose of the work described here is to define the role of the normal microflora in the expression of toxin A receptors using the axenic mouse as a reference. For comparison, the influence of the presence of the microflora on the binding of cholera toxin to receptor preparations was also determined.

* Corresponding author.

MATERIALS AND METHODS

Animals. C3H/HeJ axenic adult mice were reared in a Trexler-type isolator (La Calhene, Velizy, France) and fed ad libitum a standard rodent diet (UAR, Villemoisson, France). Conventional C3H/HeJ mice (derived from axenic mice) were reared under conventional conditions and fed the same diet as was given to axenic animals.

Intestinal brush border membranes. Brush border membranes were prepared essentially as described by Kessler and co-workers (12) with minor modifications (3). Animals were killed by cervical dislocation, and the intestine was quickly removed, washed with saline, and everted with a plastic rod. The mucosae of at least five animals were separated from the intestinal walls by gentle mixing during 6 min in a Waring blender in 30 ml of 10 mM HEPES (*N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–7 mM *n*-butylamine adjusted to pH 7.4 with 0.5 M maleic acid (HMBA buffer) which contained 5 mM neutralized EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 100 mM sorbitol. After filtration, the mucosal suspension was diluted with sorbitol to reach the final concentration of 10 mM HMBA buffer, pH 7.4, and 500 mM sorbitol and thoroughly (full speed) homogenized in the blender for 4 min. The homogenate was then treated with 20 mM $MgSO_4$ and allowed to rest for 20 min at 4°C. The suspension was then centrifuged once at 3,000 × *g* for 15 min, followed by a second run of the supernatant (supernatant 1) at 28,000 × *g* for 30 min. The pellet (final vesicle preparation) containing a brush border membrane-enriched fraction was then suspended in HMBA buffer–500 mM sorbitol and stored in liquid nitrogen until the day of the experiment (the supernatant [supernatant 2] was discarded). The experiment was run in parallel with conventional and axenic mice.

Analytical determinations. The protein concentration of brush border membranes was determined by the method of Lowry et al. (14). Sucrase activity was determined by the

TABLE 1. Purification of intestinal brush border vesicles from axenic and conventional mice

Fraction[a]	Protein (mg/ml) in mouse membranes[b]		Sucrase activity (nmol/min per mg) in mouse membranes[b]	
	Conventional	Axenic	Conventional	Axenic
Homogenate	4.0 ± 0.1 (100)	4.0 ± 0.3 (100)	57 ± 1 (100)	42 ± 2 (100)
Supernatant 1	3.2 ± 0.2 (76)	3.5 ± 0.3 (83)	46 ± 2 (61)	35 ± 2 (69)
Supernatant 2	2.5 ± 0.1 (59)	2.8 ± 0.4 (66)	23 ± 1 (24)	17 ± 2 (27)
Final vesicle preparation	8.9 ± 0.3 (8)	8.7 ± 0.7 (8)	200 ± 13 (27)	147 ± 2 (30)

[a] See Materials and Methods for definitions of fractions.
[b] The numbers in parentheses are the percentages of starting homogenate protein or sucrase.

measurement of liberated glucose during hydrolysis of sucrose using the glucose oxidase peroxidase reagent (7).

Toxins and immunoassays for toxin quantitation. Toxin A used in the test (produced by *C. difficile*) was crude. It was obtained from dialysis cultures of *C. difficile* as previously described (17) and stored at −20°C until use. Cholera toxin was purchased from Sigma Chemical Co., St. Louis, Mo.

Toxin A quantities were determined by using an enzyme-linked immunosorbent assay (ELISA) procedure previously described (17). Only toxin A is revealed by this test. Cholera toxin was quantified by using a similar ELISA technique. The immunological reagents use for cholera were sheep immunoglobulin G anticholera toxin and rabbit immunoglobulin G (kindly donated by A. Dodin, Pasteur Institute, Paris, France). They were obtained after a hyperimmunization with purified cholera toxin.

Binding of toxins to brush border membranes. The binding of toxins to membranes was performed at 4°C by a modification of a previously described procedure using brush border preparations (13) in 1-ml plastic tubes previously saturated with 20 mg of bovine serum albumin per ml. Membrane preparations were quickly thawed and diluted to give the desired protein concentration in 0.1 M Tris buffer, pH 7.2, containing 50 mM sodium chloride, 500 mM sorbitol and 2% bovine serum albumin (Fraction 5; Sigma). Solutions containing the toxin were added, and after a 15-min incubation at 4°C, the mixtures were centrifuged at 28,000 × *g* for 5 min. All binding reactions were run in triplicate. The supernatants containing the residual toxin not fixed to the membranes were quantitated by using an ELISA previously described (17). To increase the precision of the ELISA for residual toxins, and in order to be in the linear portion of the reference curve, three dilutions of each sample were performed. Furthermore, comparison of the binding capability of membranes from axenic and conventional mice was accomplished by having all samples (including reference mixtures) at a given concentration of either membranes or toxin in the same microdilution plate.

In vivo enterotoxin activity. Axenic and conventional mice were inoculated intragastrically with 1 ml of a supernatant of a *C. difficile* culture containing 25 μg of toxin A per ml. We determined the mortality rate of axenic and conventional animals at 24 h after injection.

Statistics. All the statistical comparisons were done using the Student *t* test, except for mortality rates, for which the chi-square test was used.

RESULTS

A comparison of three separate purifications of brush border membrane preparations from axenic and conventional mice prepared under identical conditions is shown in Table 1. At each step the quantities of proteins from axenic and conventional mice were not significantly different. Sucrase activity was used as a brush border marker for membrane purification (12). For the whole procedure, the specific sucrase activities of the membranes from axenic mice were about 25% lower than those from membranes from conventional mice; however, the extent of purification under identical conditions was approximately the same.

The fixation of toxin A to enriched brush border membrane fraction from axenic and conventional mice was determined by using a fixed quantity of toxin A and various amounts of membranes (Fig. 1). Toxin A was found to bind to a significantly greater extent to preparations from conventional mice than to preparations from axenic mice in the range of membrane concentrations of 20 to 80 μg/ml. At 40 μg of protein, for example, about 20 ng of toxin A was bound to membranes from conventional mice as compared with 7 ng for those from axenic mice ($P < 0.001$). Above 100 μg/ml, the amount of bound toxin approached the theoretical maximum of 30 ng which was added to the test mixtures and the differences between the two preparations in binding were not as pronounced. When the amount of toxin A added to a fixed amount of membrane was varied from 20 to 160 ng/ml, a great difference between conventional and axenic membranes was evident for the maximum binding capacity of the membrane (Fig. 2). Membranes from axenic mice were saturated at toxin A concentrations of about 60 ng, whereas membranes from conventional mice still did not show saturation at a concentration as high as 160 ng/ml. The maximum amount of toxin A bound to membranes from axenic mice was about 30 ng compared with over 80 ng for membranes

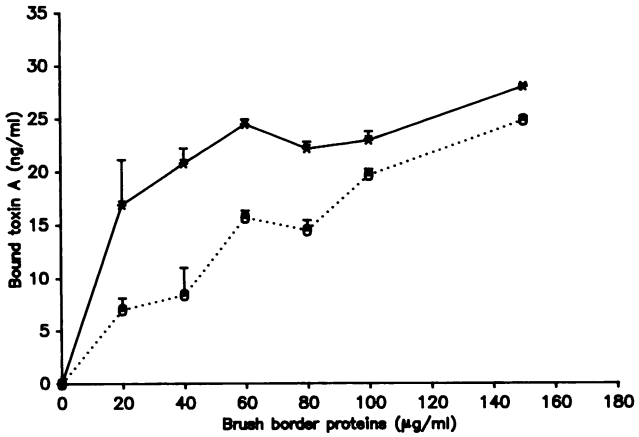

FIG. 1. Binding of *C. difficile* toxin A to mouse brush border membranes. The influence of membrane concentration is shown. · · · , Axenic mice; ——, conventional mice.

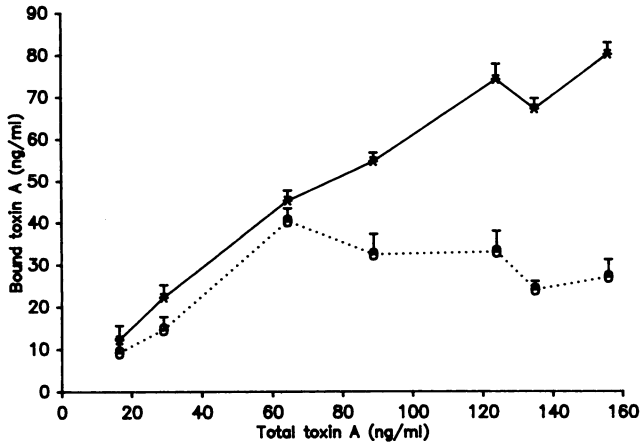

FIG. 2. Binding of *C. difficile* toxin A to mouse brush border membranes. The influence of toxin A concentration is shown. · · · , Axenic mice; ——, conventional mice.

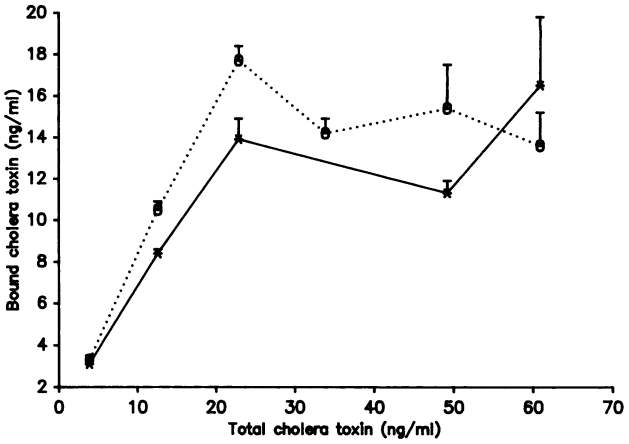

FIG. 4. Binding of cholera toxin to mouse brush border membranes. The influence of toxin concentration is shown. · · · , Axenic mice; ——, conventional mice.

from conventional mice ($P < 0.001$). It is important to note that the amount of toxin fixed at low toxin concentrations (<60 ng) was not significantly different between the preparations from axenic and conventional mice.

The binding of cholera toxin measured under the same experimental conditions, using the same preparations as those used in Fig. 1 and 2, showed that there was little difference in the abilities of membranes from either conventional or axenic mice to bind cholera toxin (Fig. 3). A maximum of 20 ng of cholera toxin was bound by 20 μg of membranes from either axenic or conventional mice. Similarly, a saturation experiment revealed that membranes (10 μg) from both sources were saturated at values of greater than about 25 ng of cholera toxin per ml (Fig. 4). Differences in the amount of cholera toxin bound were not significant between the two preparations.

Two groups of 20 mice, conventional and axenic, were inoculated intragastrically with about 25 μg of toxin A. At 24 h, the mortality reached 55% for conventional mice and 25% for axenic mice. The difference was statistically significant ($P < 0.05$). Furthermore, the intestines from dead mice were strongly hemorrhagic and similar in appearance to those from *C. difficile*-infected mice.

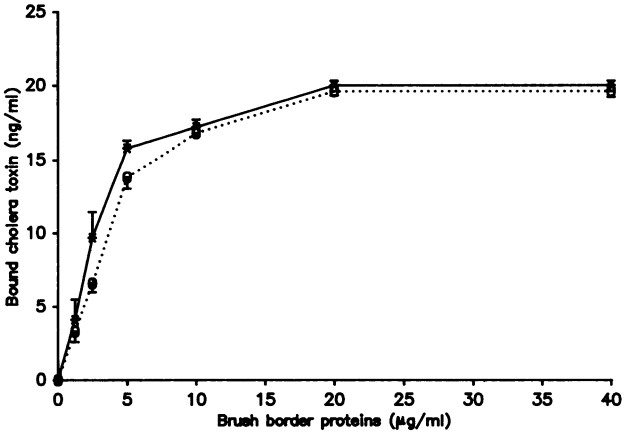

FIG. 3. Binding of cholera toxin to mouse brush border membranes. The influence of membrane concentration is shown. · · · , Axenic mice; ——, conventional mice.

DISCUSSION

For this study on the influence of the intestinal flora on the binding of *C. difficile* toxin A to intestinal epithelial receptors, we used enriched brush border membrane preparations rather than crude brush border membrane preparations as described earlier (13). The membrane vesicles prepared by this technique have more than 95% of the mucous surface of the brush border exposed to the incubation medium, thus minimizing nonspecific adhesion to the serous side of the membranes (12). We have shown that it is possible to obtain good reproducibility in purification of successive batches of membranes. Moreover, there were no fundamental differences in protein content and sucrase activities between material obtained from intestines from axenic and conventional mice. These similarities have facilitated standardization of our working parameters and allowed direct comparisons of the two types of intestinal preparations. Mouse intestines rather than ceca were used as a source of receptors because cholera disease pathology is confined to the intestines, while *C. difficile* pathology is mainly confined to cecum where *C. difficile* multiplies. Exposure of the intestine to toxin A will cause pathology there as well (the rabbit ileal loop is used as a test for toxin A activity). It is not unreasonable to postulate that the properties of the intestinal receptor and the cecal receptor are similar.

Under the same test conditions using small amounts of membranes, more *C. difficile* toxin A was bound to membranes from conventional mice than to those from axenic mice. These data could be interpreted in two ways: the conventional membranes had a higher number of receptors on their surfaces or the receptors had a higher affinity. However, when the membrane protein was held constant and the toxin was increased, we found virtually no difference between membranes from axenic and conventional mice at low toxin concentrations but a profound difference in the amount of toxin required to saturate the two preparations. These last findings suggest that the differences in binding to membranes from axenic and conventional mice can be only attributed to an appreciably greater number of toxin A receptors on the membranes from conventional mice. The difference in the number of receptors between axenic and holoxenic mice is only about threefold. However, *C. difficile* toxin A is produced in excess because it is found unbound in cecum contents and in stool. Thus, the effect of toxin A is

dependent on total fixation. A threefold increase in the number of receptors will be related to an increase in fixation, and this could lead to an increase in pathology.

With identical techniques and preparations, no differences were observed in binding characteristics of cholera toxin to membranes from axenic or conventional mice. Thus, the presence and/or the action of the intestinal flora increases the number of *C. difficile* toxin A receptors at the enterocyte surface but has no apparent influence on the receptors to cholera toxin. It is well known that the two receptors are different (9, 13), and one can imagine that bacterial activities may stimulate synthesis of one type of receptor rather than the other. The effect due to the intestinal flora may be an indirect one and could be mediated by change in intestinal cell turnover rate, modification in bile acid secretions, or any other effect mediated by flora on the host.

With a greater number of toxin A receptors, one would anticipate that conventional animals would be more sensitive to *C. difficile* toxin A than are axenic animals, and this is what we found. The situation with an infection of toxinogenic *C. difficile* is, however, more complex because the normal bacterial flora acts as a barrier to establishment and proliferation of the pathogen (6). On the other hand, the increased number of receptors could act as an aggravating factor for *C. difficile* pathology in humans when large quantities of antibiotics which destroy the barrier flora are used.

ACKNOWLEDGMENTS

We thank F. Alvarado and P. Raibaud for their helpful advice. We thank A. Dodin for his help in cholera toxin quantitation by immunoassays.

LITERATURE CITED

1. **Bartlett, J. G.** 1979. Antibiotic-associated-pseudomembranous colitis. Rev. Infect. Dis. **1**:530–538.
2. **Bartlett, J. G., A. B. Onderdonk, R. L. Cisneros, and D. L. Kasper.** 1977. Clindamycin-associated colitis due to toxin-producing species of *Clostridium* in hamsters. J. Infect. Dis. **136**:701–705.
3. **Brot-Laroche, E., M. A. Serrano, B. Delohomme, and F. Alvarado.** 1986. Temperature sensitivity and substrate specificity of two distinct sodium activated D-glucose transport systems in guinea pig jejunal brush border membrane vesicles. J. Biol. Chem. **261**:61–76.
4. **Chang, T. W., J. G. Bartlett, S. L. Gorbach, and A. B. Onderdonk.** 1978. Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect. Immun. **20**:526–529.
5. **Corthier, G., F. Dubos, and P. Raibaud.** 1985. Modulation of cytotoxin production by *Clostridium difficile* in the intestinal tracts of gnotobiotic mice inoculated with various human intestinal bacteria. Appl. Environ. Microbiol. **49**:250–252.
6. **Dabard, J., F. Dubos, L. Martinet, and R. Ducluzeau.** 1979. Experimental reproduction of neonatal diarrhea in young gnotobiotic hares simultaneously associated with *Clostridium difficile*, and other *Clostridium* strains. Infect. Immun. **24**:7–11.
7. **Dahlqvist, A.** 1964. Method for assay of intestinal disaccharidases. Anal. Biochem. **7**:18–25.
8. **Ebright, J. R., R. Fekety, J. Silva, and K. H. Wilson.** 1981. Evaluation of eight cephalosporins in hamster colitis model. Antimicrob. Agents Chemother. **19**:980–986.
9. **Fishman, P. H.** 1982. Role of membrane gangliosides in the binding and action of bacterial toxins. J. Membr. Biol. **69**:85–97.
10. **George, W. L., R. D. Rolfe, and S. M. Fingold.** 1982. *Clostridium difficile* cytotoxin in feces of patients with antimicrobial agent-associated diarrhea in miscellaneous conditions. J. Clin. Microbiol. **15**:1049–1053.
11. **George, W. L., R. D. Rolfe, G. K. M. Harding, R. Klein, C. W. Putnam, and S. M. Fingold.** 1982. *Clostridium difficile* and cytotoxin in feces of patients with antimicrobial agent associated pseudomembranous colitis. Infection **10**:205–207.
12. **Kessler, M., O. Acuto, C. Storelli, H. Murer, M. Müller, and G. Semenza.** 1978. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Biochim. Biophys. Acta **506**:136–154.
13. **Krivan, H. C., G. F. Clark, D. F. Smith, and T. D. Wilkins.** 1986. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glucoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect. Immun. **53**:573–581.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Lyerly, D. M., D. E. Lockwood, S. H. Richardson, and T. D. Wilkins.** 1982. Biological activities of toxins A and B of *Clostridium difficile*. Infect. Immun. **47**:349–352.
16. **Lyerly, D. M., K. E. Saum, D. K. McDonald, and T. D. Wilkins.** 1985. Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect. Immun. **47**:349–352.
17. **Mahe, S., G. Corthier, and F. Dubos.** 1987. Effect of various diets on toxin production by two strains of *Clostridium difficile* in gnotobiotic mice. Infect. Immun. **55**:1801–1805.
18. **Taylor, N. S., G. M. Thorne, and J. G. Bartlett.** 1981. Comparison of two toxins by *Clostridium difficile*. Infect. Immun. **34**:1036–1043.
19. **Toothaker, R., and G. W. Elmer.** 1984. Prevention of clindamycin-induced mortality in hamsters by *Saccharomyces boulardii*. Antimicrob. Agents Chemother. **26**:552–556.
20. **Triadafilopoulos, G., C. Pothoulakis, M. J. O'Brien, and J. T. Lamont.** 1987. Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterology **93**:273–279.